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Sound evoked fos-like immunoreactivity in the big brown bat

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ABSTRACT

Most bat species have highly developed audio-vocal systems, which allow them to adjust the features of echolocation calls that are optimized for different sonar tasks, such as detecting, localizing, discriminating and tracking targets. Furthermore, bats can also produce a wide array of social calls to communicate with conspecifics. The acoustic properties of some social calls differ only subtly from echolocation calls, yet bats have the ability to distinguish them and reliably produce appropriate behavioral responses. Little is known about the underlying neural processes that enable the correct classification of bat social communication sounds. One approach to this question is to identify the brain regions that are involved in the processing of sounds that carry behavioral relevance. Here, we present preliminary data on neuronal activation, as measured by c-fos expression, in big brown bats (*Eptesicus fuscus*) exposed to either social calls, echolocation calls or kept in silence. We focused our investigation on five relevant brain areas; three within the canonical auditory pathway (auditory cortex, inferior colliculus and medial geniculate body) and two that are involved in the processing of emotive stimulus content (amygdala and nucleus accumbens). In this manuscript we report c-fos staining of the areas of interest after exposure to conspecific calls. We discuss future work designed to overcome experimental limitations and explore whether c-fos staining reveals anatomical segregation of neurons activated by echolocation and social call categories.

Introduction

Echolocating bats are auditory specialists that produce sonar signals and process acoustic information carried by returning echoes to represent the location and features of objects in their surroundings (Griffin, 1958; Popper & Fay, 1995; Thomas et al., 2003). Acoustic information that the bat obtains from its surroundings comes not only from self-generated echo returns, but also from echolocation and social communication sounds produced by neighboring conspecifics. As such, the mix of echolocation and social communication sounds creates a cocktail party-like environment (Cherry, 1953; Lewicki et al., 2014) in which bats operate. Many bat communication and echolocation calls contain overlapping acoustic features, which these animals must distinguish to successfully extract behaviorally relevant information. The big brown bat (*Eptesicus fuscus*), an aerial hawking insectivore, emits frequency modulated (FM) sonar signals to track and intercept flying insect prey. This species also produces a variety of communication calls in social encounters (both appeasement calls and aggressive calls), mother-pup interactions, and foraging (Gadziola et al., 2016; Monroy et al., 2011; Wright et al., 2013).

We aim to understand the neural mechanisms that enable the recognition and classification of species-specific sounds to guide appropriate behavioral responses. One step towards unraveling the neural substrates of social sound processing is to evaluate the activation of different brain areas while bats passively listen to social and echolocation sounds. In this study, we use the expression of the early gene c-fos to measure neuronal activation in big brown bats exposed to echolocation sounds, an assortment of conspecific social sounds, or kept in silence. The early gene c-fos is a transcription factor that is newly syntheszised 30–90 min post-depolarization of neurons and has been used in many species as a marker for neuronal activation (Del Mar Díaz-González et al., 2019; Hartline et al., 2017; Monbureau et al., 2015; Pena et al., 2017; Smith et al., 2019; Xu et al., 2019). For this report, we targeted five different brain areas of interest. We quantified the number of c-fos positive cells in three areas that are part of the central auditory pathway: the central nucleus of the Inferior Colliculus (IC) and the Auditory Cortex (AC) and the medial geniculate body (MGB). The IC is an auditory hub that receives ascending input from brain stem nuclei and through the MGB sends inputs to AC, a cortical area implicated in complex sound processing (Amunts et al., 2012). Electrophysiological

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studies in these brain areas of the bat's brain reveal neural selectivity to social calls (Kanwal & Rauschecker, 2007; Salles et al., 2020). The other two areas of interest are part of the limbic system, the basolateral amygdala (Amy), implicated in fear and emotive processing (Phelps & LeDoux, 2005); and the Nucleus Accumbens (NAc), implicated in reward and sound categorization (Goto & Grace, 2008; Lim et al., 2014). In the big brown bat, electrophysiological recordings in the amygdala have demonstrated that single neurons show selectivity to different communication calls (Gadziola et al., 2016), yet no studies to date have evaluated the role of the Nucleus Accumbens in social call processing.

C-fos expression has been used in bats to study neural activation by sound stimuli; however, no research has yet attempted comparisons across different brain regions and call categories (Echolocation vs. Social calls). In Mexican free-tailed bats, *Tadarida brasiliensis*, c-fos expression was used to map the neural activation patterns in spontaneously echolocating animals (Schwartz & Smotherman, 2011). Previous studies in big brown bats exposed to playbacks of echolocation calls showed increases in the number of c-fos positive cells, as compared to silence (Jen et al., 1997; Qian & Jen, 1994). Here, we extend this line of investigation and compare neural activation by social and echolocation sound stimuli in five targeted brain regions of the big brown bat.

Our hypothesis is that the discrimination of sounds that carry different information arises from differential activation of neurons in the central auditory pathway (inferior colliculus, medial geniculate body and the auditory cortex) and brain areas involved in affective stimulus processing (limbic system). We expected that both the IC, MGB and AC would show extensive activation to all call categories but also predicted that we would see differences in the number of activated cells in the Amy and NAc. In this study, we found that bats exposed to playbacks of conspecific vocalizations have higher numbers of c-fos positive cells than those kept in silence. Yet, with this approach no differences were observed in the number of neurons activated by echolocation call and social call exposure. We present this report to share preliminary data and to highlight pitfalls and solutions, which can guide next steps to exploit this technique further to dissect subregional differences in cell activation that may arise from the exposure to different call categories.

Methods

Animals

The animals were retrieved from an exclusion site in Maryland under collection permit #55440. The animals were housed in rooms with humidity set at 50% and temperature set at 25 °C. Seven females and four males were used for this experiment, with at least one male per group. Four animals were used for the social sounds group (DB8, GY12, OR3, PK6), four animals for the echolocation group (DB23, G1, R5, W28) and three animals for the silence group (OJ, 13CC, OCE6). All protocols for animal research were approved by the Animal Care and Use Committee of Johns Hopkins University. Protocol number: BA20A65.

Playbacks

Each bat was habituated to a ventilated sound-proof chamber (manufactured by BRS/LVE) for an hour each day, for four consecutive days. On the day of exposure, the bats were placed in the sound-proof chamber, and depending on the group the animal was assigned to, a randomized set of either social calls, echolocation calls, or no calls (silence) was presented for one hour (Fig. 1) (Cody et al., 1996; Monbureau et al., 2015). Each separate call from each category was played in a random order (not as a sequence) to each bat and played at a rate of one individual call per second, at 70 dB SPL using a customized LabView code through a National Instruments board. Bat signals were selected from data from free-flying bats, recorded in an anechoic flight room in the laboratory. Social calls were recorded from pairs of bats flying

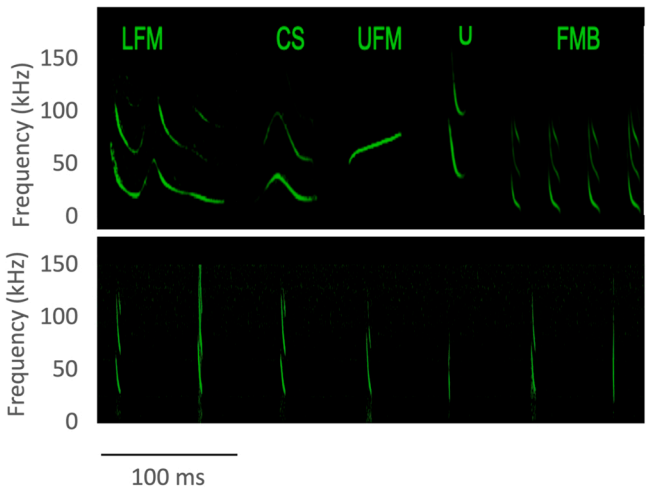

Fig. 1. Spectrogram of the social call and echolocation call playbacks. The top panel is a set of social calls used for playbacks during the day of exposure. The social call types used included long frequency-modulated (LFM), chevron-shaped (CS), upward frequency-modulated (UFM), U-shaped (U), and frequency-modulated bout (FMB), as described in Wright et al. 2013. The bottom panel is a set of echolocation calls used for playbacks during the day of exposure.

together while echolocation calls were recorded in trials when bats flew alone. The playbacks were transmitted by a custom ultrasound electrostatic loudspeaker (1 cm diam), and the playbacks were passed through a filter, as described in Luo et al., 2018, to obtain a flat frequency response. The speaker was powered by a Krohn-Hite 7500 DC amplifier.

Histology

The bats were sacrificed immediately following exposure and were perfused with 4% PFA. The brains were extracted and then transferred to 15% sucrose for 24 h and then 30% sucrose for 24 h. Each brain was then sectioned in 50 μm slices using a cryostat (Leica CM1860). The slices were collected in phosphate buffered saline (PBS 1M) and free-floating DAB protocol was carried out as follows:

The slices were permeabilized in PBS 1M - Triton 0.03% for 10 min on a shaker. Then, they were incubated for 30 min with normal horse serum blocking solution, 2.5% (Vector Laboratories S-2012–50). Then, the slices were incubated overnight at 4 °C with mouse monoclonal Anti-Fos/c-Fos Antibody (E-8 sc-166940) in normal horse serum at 1:250 dilution. The next day, the slices were washed for 5 min in phosphate buffer (PB 1M). Afterwards, the slices were incubated for 45 min at room temperature with horse anti-mouse/rabbit IgG antibody (Universal), Biotinylated, R.T.U. (BP-1400–50). The slices were washed again for 5 min in PB and incubated with VECTASTAIN® Elite® ABC Universal PLUS (Kit No. PK-8200) at room temperature for 30 min and washed in PB for 5 min. Finally, they were incubated for 1 min in freshly prepared ImmPACT DAB EqV Substrate (Reagent 1 + Reagent 2; Kit No. PK-8200). The slices were rinsed in tap water, mounted, and dried for an hour before sealing over with Permount (Fisher SP15–5000).

For a control we acquired images from the somatosensory cortex (S1) of each bat and followed the same analysis as the areas of interest. All bats were exposed to the same environment, and thus we do not expect changes in S1 activation for the different groups.

Five images from each area were acquired for each animal from adjacent slices with a 20/0.40 of an AMScope microscope and AMScope MU500 camera acquisition system, with the exception of Nucleus Accumbens in bat OR3 for which the frontal part of the brain could not be sliced properly, preventing c-fos positive cell counts in NAc of this animal.

Cresyl violet staining as described in the Cold Spring Harbor methods

(Paul et al., 2008) was used to establish anatomical landmarks from adjacent brain slices previously stained for c-fos (Fig. S1) and these were compared with the unpublished big brown bat brain atlas from Dr. Ellen Covey's Bat Lab at the University of Washington.

Data analysis and statistics

The images were analyzed using FIJI software following a custom automatization of cell counts, and each image was manually inspected for accuracy to corroborate the detected cells. The regions of interest used were the same size for all areas, 300 × 225 μm. The custom code for detection runs the following steps: run("8-bit"); run("Enhance Contrast.", "saturated=0.3"); run("Subtract Background.", "rolling=50 light"); run("Enhance Contrast.", "saturated=0.3"); setAutoThreshold ("Default dark"); //run("Threshold."); setThreshold(210, 255); //setThreshold(188, 255); run("Convert to Mask"); run("Convert to Mask"); run("Watershed"); run("Analyze Particles.", "size=300–3500 circularity=0.20–1.00 show=Outlines clear summarize in_situ"). Statistical analyses were carried out with Graphpad Prism 9. The data for each animal were tested for normality with the Shapiro-Wilk test. Nested ANOVAs and Fisher's LSD tests were performed to compare the effect of stimulus conditions on neural activation in each brain area.

Results

The numbers of c-fos positive cells were counted in three different slices from each brain area of interest obtained from each animal in every group. Fig. 2 shows example images from the five areas that were examined (AC, MGB, IC, Amy and NAc) for animals in each of the three exposure conditions (Social Calls, Echolocation, Silence). We also counted the number of c-fos positive cells in S1 as a control across groups and animals; all bats were exposed to the same environment, and thus we did not expect differences in the number of c-fos positive cells in this brain region. Our results (Fig. 3) show that there was a significantly greater number of c-fos positive cells in the sound groups for the Auditory cortex (AC Echolocation vs. Silence $P < 0.05$, $t = 3.72$, $DF = 9$; AC Social vs. Silence $P < 0.05$, $t = 3.343$, $DF = 9$), while there was no significant difference between the bats exposed to the different sounds (AC Social vs. Echolocation $P = 0.603$, $t = 0.5389$, $DF=9$). We also observed this result in the IC, the MGB and the Amygdala; there was no significant difference in the number of c-fos positive cells between the groups exposed to echolocation or social calls (IC Social vs. Echolocation $P = 0.1894$, $t = 1.434$, $DF = 8$; MGB Social vs. Echolocation $P = 0.2958$, $t = 1.062$, $DF = 33$; Amy Social vs. Echolocation $P = 0.8413$, $t = 0.2068$, $DF = 8$), but there was a significant difference between the groups exposed to sounds and the silence group (IC Social vs. Silence $P < 0.05$, $t = 3.651$, $DF = 8$; IC Echolocation vs. Silence $P < 0.05$, $t = 2.323$, $DF=8$; MGB Social vs. Silence $P < 0.0001$, $t = 5.226$, $DF = 33$; MGB Echolocation vs. Silence $P < 0.001$, $t = 4.206$, $DF = 33$; Amy Social vs. Silence $P < 0.05$, $t = 2.808$, $DF = 8$; Amy Echolocation vs. Silence $P < 0.05$, $t = 3.007$, $DF = 8$). In the Nucleus Accumbens, there was also no difference in the number of c-fos positive cells between the sound exposure groups (NAc Social vs. Echolocation $P = 0.081$, $t = 2.038$, $DF = 7$), and similar to the other areas, there was a significant difference in the number of c-fos positive cells between the echolocation and silence groups (NAc Echolocation vs. Silence $P < 0.05$, $t = 4.448$, $DF = 7$). In NAc, there was no significant difference between the social and silence groups; however, a trend showed a higher number of c-fos positive cells in the social call group (NAc Social vs. Silence $P = 0.0588$, $t = 2.254$, $DF = 7$). This comparison only included three animals for each group, as the portion of the brain that encompases the Nucleus Accumbens in bat OR3 in the social group was lost during slicing, which could have led to the lack of significance in the comparison. As expected, S1 showed no significant difference in c-fos positive cells across groups (S1 Social vs. Echolocation $P = 0.6690$, $t = 0.4438$, $DF = 8$;S1; S1 Social vs. Silence $P = 0.1775$, $t = 1.479$, $DF = 8$; S1 Echolocation vs. Silence $P = 0.3168$, $t = 1.068$, $DF = 8$).

Discussion

The early transcription factor c-fos has been used widely to map neuronal activation across taxa, including mice, rats, reptiles, fish, birds and mollusks (Del Mar Díaz-González et al., 2019; Hartline et al., 2017; Monbureau et al., 2015; Pena et al., 2017; Smith et al., 2019; Xu et al., 2019). This transcription factor gene is transcribed and translated to protein approximately 30–90 min after neuronal activation, providing a reliable marker for the neurons that depolarized during a stimulus event or behavior. In the songbird, the use of c-fos mapping has enabled the identification of brain areas involved in social sound production and processing (Bailey & Wade, 2003; Riters et al., 2004). In bats, only few studies have employed the use of c-fos mapping (*E. fuscus* (Jen et al., 1997; Qian & Jen, 1994); *T. brasiliensis* (Schwartz & Smotherman, 2011)), and none before this report have compared activation patterns across call categories (social calls vs. echolocation calls).

Our results showed an increase in activated neurons in auditory (AC and IC) and limbic (Amy and NAc) brain regions in bats exposed to conspecific calls, as compared to animals kept in silence, thus replicating previous findings (Jen et al., 1997; Qian and Jen, 1994). Our experiment, however, did not show evidence of any differences in the number of cells activated in these areas by separate call categories (Echolocation vs. Social calls). Nevertheless, we cannot dismiss the possibility that differences in call activation patterns within and across brain regions may be masked by broad categorization of stimuli (social vs. echolocation) and/or sound exposure time. In this experiment, we used one hour of sound exposure, as had been previously adopted for canaries and guinea pigs (Cody et al., 1996; Monbureau et al., 2015), with a randomized presentation of calls for each category. Going forward we will modify the protocol to expose the animals for 30 min and allow 60 min of silence before sacrificing the animals to better capture the peak activation of c-fos, limiting potential overexposure to sound stimuli and any other potential spurious activation.

Additional considerations for future work: Other brain regions, such as prefrontal cortex and visual cortex, could be added as comparative controls in future experiment as well. Furthermore, in our next experiments, appeasement social calls, aggressive social calls, and echolocation calls will be broadcast separately to bats assigned to different exposure groups. In this report, both appeasement and aggressive calls were presented in the same category (Social calls). Thus, the social valence that we predict to evoke differential activation patterns in the Amy and NAc cannot be discerned. Furthermore, the conditions used in the experiment cannot rule out the possibility that c-fos expression was evoked specifically by conspecific vocalizations and not more generally to sounds of any category; adding conditions with white noise and heterospecific animal sounds would address this question. In our experiment, the bats were monitored with a web cam and no activity could be discerned, the bats remained mostly motionless and did not show oro-facial movements that typically accompany vocalizations. Nevertheless, in future experiments any auditory contribution from self-produced calls will be captured with ultrasound microphones. Future experiments will also investigate differential expression in subregions of the areas of interest and expand the analysis to other auditory areas (Miller & Covey, 2011) and brain areas involved in behavioral responses to social stimuli, such as the periaqueductal gray (Fenzl & Schuller, 2002) and the prefrontal cortex (Rose et al., 2021).

The use of molecular tools to study the neural substrates of bat acoustic communication has been underexplored, and it is an important step towards understanding the mechanisms of acoustic signal processing in mammals. With this report, we aimed to establish the groundwork that will enable further use of techniques to map neuronal activation patterns in response to natural sounds. Because of the rich and complex structure of bat social signals, this research has broad implications for advancing a more general understanding of natural sound

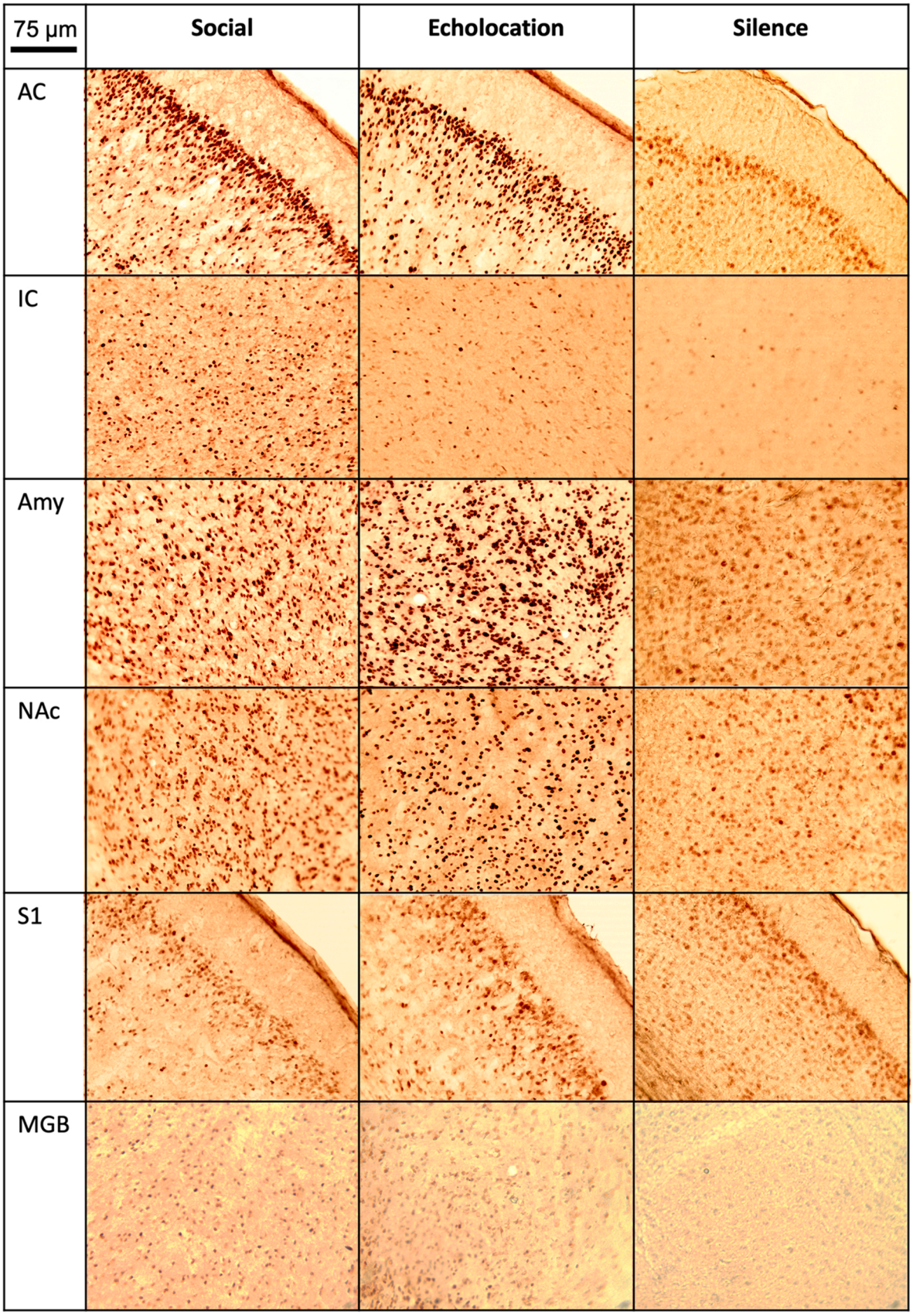

Fig. 2. Examples of c-fos staining. C-fos staining was performed for sections from the auditory cortex (AC), inferior colliculus (IC), amygdala (Amy), nucleus accumbens (NAc), somatosensory cortex (S1), and medial geniculate body (MGB) for the bats in the social calls, echolocation, and silence exposure groups.

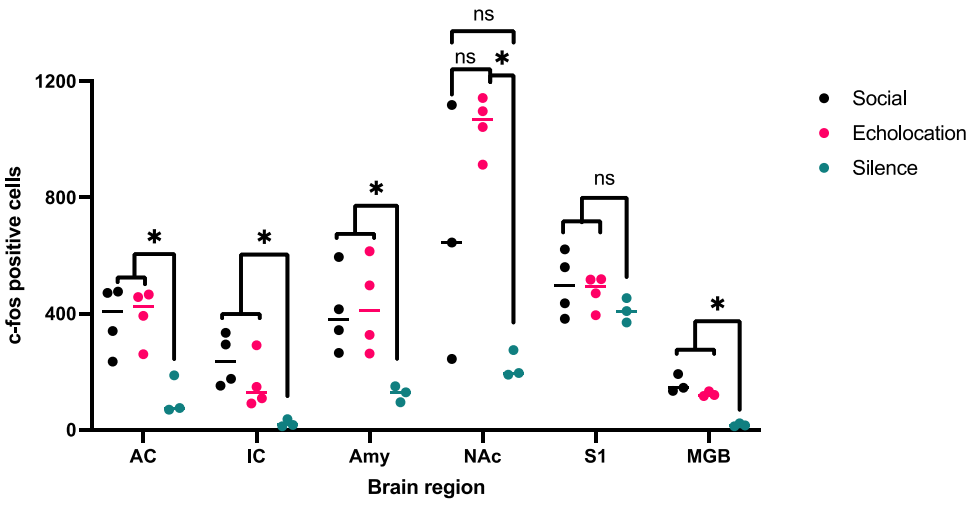

Fig. 3. Sound exposure led to a significant increase in c-fos positive cells. The number of c-fos positive cells was significantly higher in the sound groups (social and echolocation) than in the silence group for the auditory cortex (AC), inferior colliculus (IC), amygdala (Amy), nucleus accumbens (NAc), and medial geniculate body (MGB). As a control we counted the c-fos positive cells in the somatosensory cortex of the bats (S1), there is no difference in number of activated cells in each treatment for this area. Individual data points represent animals: Echolocation group, n = 4; Social group n = 4 except for MGB and NAc where n = 3; Silence n = 3. There was no significant difference in the number of c-fos positive cells between the bats in the social calls group and the bats in the echolocation group.

processing, such as speech in humans and acoustic communication signals in other animals.

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Compliance with ethical standards

We adhere to the Elsevier and the Johns Hopkins University ethical guidelines. We have no conflicts of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibneur.2022.02.005.

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